Final Report to US Department of Energy - Award No. DE-FG02-01ER63145 entitled "Improving functional analysis of genes relevant to environmental restoration via an analysis of the genome of *Geobacter sulfurreducens*".
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This project elucidated the function of a number of genes involved in electron transport and other important functions in *Geobacter sulfurreducens*. For example, one of the most important accomplishments was the elucidation of the function of two c-type cytochrome genes. Both of these genes have significant sequence similarity to "*ferA*", a gene previously proposed to encode a multi-heme *c*-type cytochrome, which co-purified with a membrane-bound Fe(III) reductase complex from *G. sulfurreducens*. Analysis of the complete genome of *G. sulfurreducens* revealed that "*ferA*" is in fact a hybrid of *omcB* and *omcC*. Both *omcB* and *omcC* encode outer-membrane *c*-type cytochromes and were expressed during growth in with acetate as the electron donor and either fumarate or Fe(III) as the electron acceptor. However, the protein, OmcB, was ~2-fold more abundant under both conditions. Disrupting *omcB* or *omcC* by gene replacement had no negative impact on growth with fumarate. The OmcB deficient mutant, however, was impaired in its ability to reduce Fe(III) both in cell suspensions and under growth conditions. In contrast, the ability of the OmcC deficient mutant to reduce Fe(III) was similar to wild type. When *omcB* was reintroduced into the OmcB deficient mutant, the capacity for Fe(III) reduction was restored in proportion to the level of OmcB production. These results suggest that OmcB has a major role in electron transport to Fe(III), but OmcC does not. OmcB is the first outer-membrane cytochrome shown to be necessary for Fe(III) reduction in *G. sulfurreducens*. These results further suggest that electron transport to the outer membrane is an important feature in Fe(III) reduction of *G. sulfurreducens*. A manuscript summarizing these results was published in the *Journal of Bacteriology*.

In collaboration with the biochemical studies conducted by Jon Lloyd, genetic studies were carried out to elucidate the function of a 9.6 kDa periplasmic cytochrome from *G. sulfurreducens*. Acetate-dependent Fe(III) reduction was significantly inhibited in both growing cultures and cell suspensions of a knock-out mutant, which no longer expressed the 9.6 kDa cytochrome gene, *cycP*. In contrast, the mutation had no impact on Fe(III) reduction with hydrogen as the electron donor or the reduction of fumarate with either acetate or hydrogen. When *cycP* was expressed *in trans* the full capacity for Fe(III) reduction with acetate was restored. Cell suspensions of the mutant could not reduce U(VI) with acetate as the electron donor, but when hydrogen was the electron donor U(VI) was reduced at the same rate as in the wild type strain. Similar results were obtained with the humic analog AQDS. This is the first report of the targeted disruption of a gene involved in the reduction of Fe(III), U(VI) or extracellular quinones in *G. sulfurreducens*. These results demonstrate that portions of the electron transport pathways for acetate-dependent Fe(III), U(VI), and extracellular quinone reduction are significantly different than those for hydrogen-dependent reduction of these electron acceptors. When coupled with previous studies on other electron transport proteins in

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G.sulfurreducens, the results suggest that CycP serves as an intermediary electron carrier involved in electron transport from acetate to terminal Fe(III) reductases in the outer membrane, and is also involved in the transfer of electrons from acetate to U(VI) and humics. A manuscript summarizing these findings was published in *Biochemical Journal.*

Heme-staining of SDS-PAGE gels loaded with membrane preparations from cells grown with Fe(III) or fumarate as the electron acceptor revealed that a cytochrome with a molecular weight of ca. 36 kDa was highly abundant in Fe(III)-grown cells, but not detected in fumarate-grown cells. Analysis of the purified protein with mass spectrometry lead to the identification of the gene for this cytochrome, which was predicted to be a di-heme cytochrome with ca. 60% identity to cytochromes from *Rhodobacter capsulatus* and *Pseudomonas aeruginosa* that have been annotated as hydrogen peroxide oxidoreductases. However, when the gene for this cytochrome was deleted from *G. sulfurreducens*, there was no impact on the ability to survive oxidative stress, but the capacity for Fe(III) and U(VI) reduction was lost. The mutant reduced fumarate as well as wild type. Complementation *in trans* restored the capacity for metal reduction. Current information suggests that this cytochrome, designated MacA (membrane associated cytochrome A), is localized in the periplasm, but associated with the inner membrane. These results suggest that MacA is a key intermediary electron transport component in electron transfer to metals in *G. sulfurreducens*. This functional analysis not only adds to the understanding of electron transfer in *G. sulfurreducens*, but also improves the annotation of genes for similar cytochromes in other organisms which may have *in vitro* peroxidase activity , but probably do not function as essential peroxidases *in vivo*. A manuscript summarizing these results was published in the *Journal of Bacteriology.*

One novel aspect of the physiology of *G. sulfurreducens* that was revealed from the genome was the potential for growth with oxygen serving as the sole electron acceptor. All members of the *Geobacteraceae* have previously been reported to be strict anaerobes. However, analysis of the genome under this grant identified not only genes for tolerating oxidative stress, but also a putative terminal cytochrome c oxidase, suggesting that *G. sulfurreducens* might have the ability to utilize oxygen as a terminal electron acceptor. Evaluation of various growth conditions and medium types failed to result in growth of *G. sulfurreducens* until after several months it was found that if *G. sulfurreducens* was pre-grown under anaerobic conditions with a small amount of an alternative electron acceptor , such as fumarate, then cells could be grown with oxygen as the sole electron acceptor after the fumarate was depleted. *G. sulfurreducens* grew best with oxygen levels one-half to one-fourth atmospheric levels. A knockout mutation of the putative cytochrome oxidase eliminated the capacity for growth on oxygen without affecting growth on anaerobic electron acceptors. These findings provide an explanation for how *Geobacter* species are able to survive at low levels in aerobic aquifers and then rapidly respond to the development of anoxic conditions during bioremediation. A manuscript summarizing these studies was published in *Applied and Environmental Microbiology.*

The initial analysis of the complete genome of *Geobacter sulfurreducens* was completed and published in *Science*. Many of the inferences in this manuscript resulted from functional analyses performed under this grant.

Hydrogen is an important electron donor for metal reduction in subsurface environments, but little is known about hydrogen metabolism in *Geobacter* species. An examination of the genome of *G. sulfurreducens* revealed two operons, *hya* and *hyb*, which, based on sequence homology, appeared to encode periplasmically-oriented respiratory uptake hydrogenases. In order to assess the roles of these two enzymes in hydrogen-dependent growth, Hya- and Hyb-deficient mutants were generated by gene replacement. Hyb was found to be required for hydrogen-dependent reduction of Fe(III), anthraquinone-2,6-disulfonate (AQDS), and fumarate by resting cell suspensions and to be essential for growth with hydrogen and these three electron acceptors, whereas Hya was not. These findings suggest that Hyb is an essential respiratory hydrogenase in *G. sulfurreducens*. This is consistent with the fact that other *Geobacteraceae*, such as *Geobacter metallireducens* and *Desulfuromonas acetoxidans*, which can not use as hydrogen as an electron donor, contain Hya, but not Hyb. A manuscript summarizing these results was published in press in the *Journal of Bacteriology*.

Publications

Methé, B. A., K. E. Nelson, J. A. Eisen, I. T. Paulsen, W. Nelson, J. F. Heidelberg, D. Wu, M. Wu, N. Ward, M. J. Beanan, R. J. Dodson, R. Madupu, L. M. Brinkac, S. C. Daugherty, R. T. DeBoy, A. S. Durkin, M. Gwinn, J. F. Kolonay, S. A. Sullivan, D. H. Haft, J. Selengut, T. M. Davidsen, N. Zafar, O. White, B. Tran, C. Romero, H. A. Forberger, J. Weidman, H. Khouri, T. V. Feldblyum, T. R. Utterback, S. E. Van Aken, D. R. Lovley, and C. M. Fraser. 2003. The genome of *Geobacter sulfurreducens*: insights into metal reduction in subsurface environments. Science 302:1967-1969.

Lin, W. C., M. V. Coppi, and D. R. Lovley. 2004. *Geobacter sulfurreducens* can grow with oxygen as a terminal electron acceptor. Appl. Environ. Microbiol. 70:2525-2528.

Butler, J. E., F. Kaufmann, M. V. Coppi, C. Nunez, and D. R. Lovley. 2004. MacA, a di-heme *c*-type cytochrome involved in Fe(III) reduction by *Geobacter sulfurreducens*. J Bacteriol 186:4042-4045.

Coppi, M. V., R. O. O'Neil, and D. R. Lovley. 2004. Identification of an uptake hydrogenase required for hydrogen-dependent reduction of Fe(III) and other electron acceptors by *Geobacter sulfurreducens*. J. Bacteriol. 186:3022-3028.

Lovley, D. R., D. E. Holmes, and K. P. Nevin. 2004. Dissimilatory Fe(III) and Mn(IV) reduction. Adv. Microb. Physiol.:(in press).

Leang, C., M. V. Coppi, and D. R. Lovley. 2003. OmcB, a *c*-type polyheme cytochrome, involved in Fe(III) Reduction in *Geobacter sulfurreducens*. J Bacteriol 185:2096-2103.

Lloyd, J. R., C. Leang, A. L. Hodges Myersen, M. V. Coppi, S. Cuifo, B. Methe, S. J. Sandler, and D. R. Lovley. 2003. Biochemical and genetic characterization of PpcA, a periplasmic *c*-type cytochrome in *Geobacter sulfurreducens*. Biochem. J. 369:153-161.